PATENT COOPERATION TREAT

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
PPD 50360/WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 99/ 02652	12/08/1999	13/08/1998
Applicant		
ZENECA LIMITED et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

[ ] as suggested by the applicant.

[X] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02652

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 195 48 301 C (DUERING KLAUS DR) 27 February 1997 (1997-02-27) the whole document	1-6
A	WO 97 48719 A (BECKERMAN JANNA L ;TEXAS A & M UNIVERSITY SYST (US); ZHANG LEI (US) 24 December 1997 (1997-12-24) the whole document	1-6
A	WO 96 29392 A (UNISEARCH LTD ;KJELLEBERG STAFFAN (AU); STEINBERG PETER (AU); NYS) 26 September 1996 (1996-09-26) the whole document	2

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 November 1999	17/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRAY K. ET AL.: "Cell-to cell signaling in the symbiotic nitrogen-fixing bacterium Rhizobium leguminosarum: autoinduction of a stationary phase and rhizosphere-expressed genes" JOURNAL OF BACTERIOLOGY, vol. 178, no. 2, 1996, pages 372-376, XP002084285 the whole document	3
A	ROSEMEYER ET AL: "luxI- and luxR-homologous genes of Rhizobium etli CNPAF512 contribute to synthesis of autoinducer molecules and nodulation of Phaseolus vulgaris" JOURNAL OF BACTERIOLOGY, vol. 180, no. 4, 1 February 1998 (1998-02-01), pages 815-821, XP002084284 ISSN: 0021-9193	3
A	THROUP J. ET AL.: MOLECULAR MICROBIOLOGY, vol. 17, 1996, pages 345-56, XP002121181 cited in the application the whole document	1-6
A	ROBSON N D ET AL: "Bacterial N-acyl-homoserine-lactone-dependent signalling and its potential biotechnological applications" TRENDS IN BIOTECHNOLOGY, vol. 15, no. 11, 1 November 1997 (1997-11-01), page 458-464 XP004092668 ISSN: 0167-7799 see the whole document; esp. p.461 r. col.	1-6
A	SWIFT S ET AL: "Quorum sensing: a population-density component in the determination of bacterial phenotype" TIBS TRENDS IN BIOCHEMICAL SCIENCES, vol. 21, no. 6, 1 June 1996 (1996-06-01), page 214-219 XP004050894 ISSN: 0968-0004 cited in the application the whole document	1-6

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/02652

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 19548301 C	27-02-1997	NONE	
WO 9748719 A	24-12-1997	AU 3570997 A	07-01-1998
WO 9629392 A	26-09-1996	AU 708962 B	19-08-1999
		AU 4999696 A	08-10-1996
		BR 9607661 A	16-06-1998
		CA 2215797 A	26-09-1996
		CN 1185173 A	17-06-1998
		EP 0815201 A	07-01-1998
		JP 11502108 T	23-02-1999
		NZ 303630 A	26-01-1998

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PPD 50360/WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB99/02652	12/08/1999	13/08/1998
International Patent Classification (IPC) or national classification and IPC C12N15/31		
Applicant ZENECA LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/1999	13.11.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Surdej; P Telephone No. +49 89 2399 7334

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB99/02652

I. Basis of th r port

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-12 as originally filed

Claims, No.:

1-6 as originally filed

Drawings, sheets:

1/1 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have be n considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☒ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-6
	No:	Claims
Inventive step (IS)	Yes:	Claims
	No:	Claims 1-6

Industrial applicability (IA) Yes: Claims 1-6
No: Claims

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Reference is made to the following documents:

D1: DE 195 48 301 C (DUERING KLAUS DR) 27 February 1997 (1997-02-27)
D2: WO 97 48719 A (BECKERMAN JANNA L ;TEXAS A & M UNIVERSITY SYST (US); ZHANG LEI (US) 24 December 1997 (1997-12-24)
D3: GRAY K. ET AL.: 'Cell-to cell signaling in the symbiotic nitrogen-fixing bacterium Rhizobium leguminosarum: autoinduction of a stationary phase and rhizosphere-expressed genes' JOURNAL OF BACTERIOLOGY, vol. 178, no. 2, 1996, pages 372-376
D4: ROSEMEYER ET AL: 'luxI- and luxR-homologous genes of Rhizobium etli CNPAF512 contribute to synthesis of autoinducer molecules and nodulation of Phaseolus vulgaris' JOURNAL OF BACTERIOLOGY, vol. 180, no. 4, 1 February 1998 (1998-02-01), pages 815-821
D5: THROUP J. ET AL.: MOLECULAR MICROBIOLOGY, vol. 17, 1996, pages 345-56, cited in the application
D6: ROBSON N D ET AL: 'Bacterial N-acyl-homoserine-lactone-dependent signalling and its potential biotechnological applications' TRENDS IN BIOTECHNOLOGY, vol. 15, no. 11, 1 November 1997 (1997-11-01), page 458-464
D7: SWIFT S ET AL: 'Quorum sensing: a population-density component in the determination of bacterial phenotype' TIBS TRENDS IN BIOCHEMICAL SCIENCES, vol. 21, no. 6, 1 June 1996 (1996-06-01), page 214-219, cited in the application
D8: WOOD DW AND PIERSON LS: 'The phzI gene of Pseudomonas aureofaciens 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production' GENE, vol. 168, no. 1, 2 February 1996 (1996-02-02), page 49-53. This document is not cited in the International Search Report but it is known to the applicant (page 6, line 17 of the description).

Introduction

The application discloses methods for the protection of plants against bacterial infection and/or virus infection transmitted by bacteria, methods of enhancing interaction between a rhizobacterium and a plant and a recombinant plant genome carrying a gene to produce the bacterial pheromone N-acyl-L-

homoserine lactone in plants.

Re Item IV

Lack of unity of invention

1. The application discloses an alternative method to protect plants against pathogens using the expression of bacterial pheromone N-acyl-L-homoserine lactones (or analogues thereof) in the said plant.
   Genes required for the expression of N-acyl-L-homoserine lactones are known from the prior art (D1, D3-D8), for example all the genes referred to in claim 4 are known. D2 discloses transgenic plants producing fungal pheromone (or pheromone analogues; from page 23, line 26 to page 26, line 19) to confer resistance to fungal infection to the said plant (for example, claims 1-2, page 5, lines 24-26; page 57, lines 21-32; page 58, lines 1-19).

2. The application contains independent claims which refer to two different technical problems. Claims 1-2 refer to a method for the protection of plants against bacterial infection and/or virus infection transmitted by bacteria and claim 3 refers to a method of enhancing interaction between a rhizobacterium and a plant. The same solution is provided to solve the two different technical problems, namely introducing into the genome of the plant by transformation the ability to synthesise N-acyl-L-homoserine lactone. However, the two different problems are not linked to each other by a special (new and inventive) technical feature in the sense of Rule 13.2 PCT since the production of transgenic plants with known genes required for the expression of the bacterial pheromone N-acyl-L-homoserine lactone (see point 1) is obvious considering D2 in combination with any of the documents D1, D3-D8 at the priority date of the application. (Thus, it is considered that claim 5 lacks an inventive step and does not provide an inventive link). Therefore, the said problems are not so linked as to form a single general inventive concept as required by Rule 13.1 PCT.

3. **2** separate inventions are therefore defined:
   1. Claims 1-2 (completely) and 4-6 (partially): Method of protecting a plant against bacterial infection and/or virus infection transmitted by bacteria using transgenic plants producing N-acyl-L-homoserine lactones (or analogues thereof).

2. Claims 3 (completely) and 4-6 (partially): Method of enhancing interaction between a rhizobacterium and a plant using transgenic plants producing N-acyl-L-homoserine lactones.

4. In response to the invitation mailed on 17 May 2000, the applicant decided to pay additional fees for the invention 2 identified by the International Preliminary Examination Authority. Therefore, the International Preliminary Examination Report is established on the entire application as filed.

Re Item V

Reasoned statement under Article 35(2) PCT with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Novelty and inventive step (Art. 33(1) - (3) PCT)

Invention 1: Claims 1-2 (completely) and 4-6 (partially).

5. **Claims 1-2 and 4-6** are new since they refer to a method and recombinant plant genome to protect plants against pathogen infection by the expression *in planta* of acylated homoserine lactones which appear not to be disclosed in the prior art. However, the said claims do not involve an inventive step in the light of document D1 in combination with D2. Considering the said documents, the technical problem of invention 1 is to provide an alternative way to protect plant against pathogen infection.

The closest prior art D1 discloses a method to protect plants against pathogens (such as *Erwinia corotovora*, column 1, line 36) by inhibiting the pheromone acylated homoserine lactone regulated processes in microorganisms using the expression *in planta* of antibodies against acylated homoserine lactone. D2 discloses transgenic plants producing fungal pheromone to confer resistance to fungal infection to the said plant (page 5, 5th paragraph; page 57, lines 21-32; page 58, lines 1-19).

The skilled person faced with the technical problem of D1 would combine the solution disclosed in D2, because D2 discloses clearly the use of plant

transformed with genes producing pheromone to protect plants against pathogenic infection; thus, the person skilled in the art would arrive at the claimed subject-matter. Therefore, using the expression of bacterial pheromone in transgenic plant to protect the said plant against bacterial infection seems to be obvious considering D1 and D2 together and it cannot be seen where the inventive step lies in Claims 1 and 3-6.
From D4-D7, it is clear that the bacterial pheromone can be selected from the group consisting of the list of genes given in claim 4.

6. **Claim 2** is not inventive in the light of D2 and any of D3, D6 and D7. A method to protect plants against pathogens by the expression *in planta* of an analogue of acylated homoserine lactone is referred to in claim 2 of the application. D3 discloses furanone compounds (for example compound 4, page 9, paragraphs 2 and 3) to inhibit acylated homoserine lactone mediated processes in pathogen such as *Erwinia corotovora* and *Pseudomonas aeruginosa*. D7 discloses also analogues of acylated homoserine lactone and their role in the inhibition of the signalling pathway (from page 461, 2nd column, last paragraph to page 462, 2nd column, 1st paragraph). Analogues of acylated homoserine lactone are also mentioned in D6 (page 215, 1st column, 2nd paragraph and 2nd column, 1st paragraph; page 218). D2 discloses also analogues of pheromone (page 4, line 4; pages 23-29) and their use in inhibition of pheromone mediated processes in pathogen. The combination of D2 with any of D3, D6 or D7 is obvious since the person skilled in the art would have contemplate the introduction into the genome of plant by transformation of the ability to synthesise analogues of acylated homoserine lactone instead of acylated homoserine lactone. Consequently, no inventive step is acknowledged for claim 2.

Invention 2: Claims 3 (completely) and 4-6 (partially).

7. Claims 3 and 4-6 are new since they refer to method and recombinant plant genome to enhance interaction between a rhizobacterium and a plant by the expression *in planta* of acylated homoserine lactones which appear not to be disclosed in the prior art. However, the said claims do not involve an inventive step in the light of document D8 in combination with D2. The technical problem of invention 2 is to provide an alternative way to enhance interaction between a

rhizobacterium and a plant.
The closest prior art document D8 discloses that the pheromone phzI of *Pseudomonas aureofaciens* is required for the production of the antibiotic phenazine (e.g. page 51, left column, last paragraph and following page). D8 also discloses that the phenazine protects plants against pathogens (see e.g. page 49, introduction) and exogenously-provided acylated homoserine lactone is capable of restoring phenazine production to the disarmed *Pseudomonas aureofaciens* phzI strain. D2 discloses transgenic plants producing fungal pheromone to confer resistance to fungal infection to the said plant (page 5, 5th paragraph; page 57, lines 21-32; page 58, lines 1-19).

The skilled person faced with the technical problem of the application would combine the teaching of D8 with the solution disclosed in D2, because D2 discloses clearly the use of plant transformed with pheromone genes to produce pheromone in the rhizosphere and D8 discloses the enhanced plant protection by rhizobacteria which is conferred by the acylated homoserine lactone production. Therefore, using the expression of bacterial pheromone in transgenic plant to enhance interaction between a rhizobacterium and a plant seems to be obvious considering D8 and D2 together and it cannot be seen where the inventive step lies in claims 3 and 4-6.

Re Item VII
Certain defects in the international application

8. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D4 and D6 is not mentioned in the description, nor are these documents identified therein.

Re Item VIII
Certain observations on the international application

9. The technical problem of the application does not seem to be solved in the application. Although it is shown that the production of N-acyl-L-homoserine lactone by plant can induce a response in bacteria, there is no indication that plants are actually protected by the said production. Thus, the solution is not

sufficiently disclosed and there is a lack of support for **claims 1 and 2** (Art. 5 and 6 PCT).

10. **Claims 1-3 and 5** do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The following functional statements do not enable the skilled person to determine which technical features are necessary to perform the stated functions: synthesis of N-acyl-L-homoserine lactone in plant. The claims attempt to define the subject-matter in terms of the result to be achieved. The technical features necessary for achieving this result should be added. Therefore, the said claims lack clarity and the scope of the said claims is not clear (Art. 6 PCT).

11. **Claim 5** refers to a "recombinant plant genome". A genome is not an entity which can be isolated as such and cannot be claimed as such. Thus, the scope of the claim is not clear (Art. 6 PCT).

12. **Claim 2** appears not to be supported by the description. Only methods using the production of natural N-acyl-L-homoserine lactones are disclosed in the application and nowhere in the description is an indication of what an analogue of N-acyl-L-homoserine lactone might be. Therefore, there is insufficiency of disclosure and a lack of support in the description for claim 2 and the scope of the claim is not clear (Art. 5 and 6 PCT).

13. Part of **claim 5** is not supported by the description as required by Article 6 PCT, as its scope is broader than justified by the description. The reasons therefore are the following: in claim 5, it is referred to a recombinant plant genome containing a gene construct for the expression of a "response regulator" of a N-acyl-L-homoserine lactone. However, the expression of a response regulator is not sufficiently disclosed and it is not supported by the description since no response regulator are shown to be expressed in plant in the application (Art. 5 and 6 PCT).

14. In **claim 3**, the expression "enhancing interaction" is not clear since it cannot be seen what kind of interaction between a rhizobium and a plant is referred to. The scope of the said claim is therefore not clear (Art. 6 PCT).

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* PPD 50360/WO

Box No. I TITLE OF INVENTION

EXPRESSION OF BACTERIAL SIGNAL MOLECULES IN PLANT

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

ZENECA Limited
15 Stanhope Gate
London W1Y 6LN
United Kingdom

☐ This person is also inventor.

Telephone No. 01344 414365

Facsimile No. 01344 481112

Teleprinter No.

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

FRAY Rupert George
Division of Plant Science
School of Biological Sciences
University of Nottingham
Sutton Bonington Campus
Loughborough LE12 5RD
GB

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is,country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

HUSKISSON Frank Mackie
Intellectual Propery Department
ZENECA Agrochemicals
PO Box 3538
Jealott's Hill Research Station,Bracknell RG42 6YA
United Kingdom

Telephone No. 01344 414822

Facsimile No. 01344 481112

Teleprinter No.

☐ **Adress for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III P

If none of the

Name and address: *(Family name follo*
The address must include postal code and
Box is the applicant's State (that is, coun

THROUP John Peter
Smith Kline Beecham
Pharmaceuticals R&D
UP1345 South Collegeville R
PO Box 5089
Collegeville PA 19426
United States of America

State *(that is, country)* of nationality:

0011 610 983 2000
Smith Kline Beecham
US
J. P. Throup's nationality
Confirmed with JPT 25/8/99 " GB "

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

WALLACE Andrew David
Unilever Research
Colworth Laboratory
Colworth House
Sharnbrook
Bedfordshire
MK44 1LQ
GB

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GRIERSON Donald
Division of Plant Science
School of Biological Sciences
University of Nottingham
Sutton Bonington Campus
Loughborough LE12 5RD
GB

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

STEWART Gordon Sidney Anderson Bernie (Deceased)
School of Pharmaceutical Sciences
University of Nottingham
University Park
Nottingham
GB

This person is:
☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATIONS OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- [X] AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [X] EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [X] EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [X] OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [X] AL Albania .....
- [X] AM Armenia .....
- [X] AT Austria .....
- [X] AU Australia .....
- [X] AZ Azerbaijan
- [X] BA Bosnia and Herzegovina .....
- [X] BB Barbados
- [X] BG Bulgaria .....
- [X] BR Brazil .....
- [X] BY Belarus .....
- [X] CA Canada
- [X] CH and LI Switzerland and Liechtenstein
- [X] CN China .....
- [X] CU Cuba .....
- [X] CZ Czech Republic .....
- [X] DE Germany .....
- [X] DK Denmark .....
- [X] EE Estonia .....
- [X] ES Spain .....
- [X] FI Finland .....
- [X] GB United Kingdom
- [X] GD Grenada
- [X] GE Georgia .....
- [X] GH Ghana .....
- [X] GM Gambia
- [X] HR Croatia .....
- [X] HU Hungary .....
- [X] ID Indonesia
- [X] IL Israel .....
- [X] IN India .....
- [X] IS Iceland
- [X] JP Japan .....
- [X] KE Kenya .....
- [X] KG Kyrgyzstan .....
- [X] KP Democratic People's Republic of Korea .....
- [X] KR Republic of Korea .....
- [X] KZ Kazakhstan .....
- [X] LC Saint Lucia
- [X] LK Sri Lanka
- [X] LR Liberia
- [X] LS Lesotho .....
- [X] LT Lithuania
- [X] LU Luxembourg
- [X] LV Latvia
- [X] MD Republic of Moldova .....
- [X] MG Madagascar .....
- [X] MK The former Yugoslav Republic of Macedonia .....
- [X] MN Mongolia
- [X] MW Malawi .....
- [X] MX Mexico .....
- [X] NO Norway
- [X] NZ New Zealand .....
- [X] PL Poland .....
- [X] PT Portugal .....
- [X] RO Romania
- [X] RU Russian Federation .....
- [X] SD Sudan
- [X] SE Sweden
- [X] SG Singapore
- [X] SI Slovenia .....
- [X] SK Slovakia .....
- [X] SL Sierra Leone
- [X] TJ Tajikistan .....
- [X] TM Turkmenistan .....
- [X] TR Turkey .....
- [X] TT Trinidad and Tobago .....
- [X] UA Ukraine .....
- [X] UG Uganda .....
- [X] US United States of America .....
- [X] UZ Uzbekistan .....
- [X] VN Viet Nam .....
- [X] YU Yugoslavia .....
- [X] ZW Zimbabwe .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- [X] ZA South Africa .....
- [X] UA United Arab Emirates .....
- [ ] .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America**: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box No. IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "**patent of addition**," or "**certificate of addition**," or if, in Box No. V, the name of the United States of America is accompanied by an indication "**continuation**" or "**continuation-in-part**": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed**: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, **the earlier application is an ARIPO application**: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation of Box IV:
HOUGHTON Malcolm John
RICKS Michael James
WATERMAN John Richard
TIERNEY Francis John
GAAL Jozsef Christopher
KENT Lindsey Ruth
PRITCHARD Judith
HUSKISSON Frank Mackie

All of:
Intellectual Property Department
ZENECA Agrochemicals
Jealott's Hill Research Station PO Box 3538, Bracknell, Berkshire RG42 6YA, United Kingdom

Telephone: 01344 414521
Telegraphic Address: 01344 481112

Box N . VI PRIORITY CLA ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 13 August 1998	9817707.4	GB		
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): _____

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

B x No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	: 5
description (excluding sequence listing part)	: 12
claims	: 1
abstract	: 1
drawings	: 1
sequence listing part of description	:
Total number of sheets	: 20

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

HUSKISSON Frank Mackie

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet part of and does not count as a sheet of the national application.

PCT

FEE CALCULATION SHEET
Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference PPD 50360/WO

Applicant
ZENECA Limited

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . £55.00 T

2. SEARCH FEE . . . . . £638.00 S

International search to be carried out by _____
(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee
The international application contains 20 sheets.

first 30 sheets £285.00 b1

_____ x _____ = _____ b2
remaining sheets additional amount

Add amounts entered at b1 and b2 and enter total at B . . . . £285.00 B

Designation Fees
The international application contains 80 designations.

_____ x _____ = £715-00 D
number of designation fees payable *(maximum 10)* amount of designation fee

Add amounts entered at B and D and enter total at I . . . . . £1000.00 I
(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . £22-00 P

5. TOTAL FEES PAYABLE . . . . . £1715-00 TOTAL
Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☒ authorization to charge deposit account (see below)	☐ bank draft	☐ coupons
☐ cheque	☐ cash	☐ other *(specify)*:
☐ postal money order	☐ revenue stamps	

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ 101

☒ is hereby authorized to charge the total fees indicated above to my deposit account.

☒ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

D02093	12th August 1999	VKKalliecadi
Deposit Account No.	Date *(day/month/year)*	Signature

ATENT COOPERATION TRL Y

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark
Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing: 24 February 2000 (24.02.00)	
International application No.: PCT/GB99/02652	Applicant's or agent's file reference: PPD 50360/WO
International filing date: 12 August 1999 (12.08.99)	Priority date: 13 August 1998 (13.08.98)
Applicant: FRAY, Rupert, George et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

15 December 1999 (15.12.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

WO

09/762769

SOCO

P ENT COOPERATION TREA

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From the INTERNATIONAL BUREAU

To:

HUSKISSON, Frank, Mackie
Syngenta Limited
Intellectual Property Dept.
Jealott's Hill Research Station
P.O. Box 3538
Bracknell, Berkshire RG42 6YA
ROYAUME-UNI

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

Date of mailing (day/month/year) 18 April 2001 (18.04.01)	
Applicant's or agent's file reference PPD 50360/WO	**IMPORTANT NOTIFICATION**
International application No. PCT/GB99/02652	**International filing date** (day/month/year) 12 August 1999 (12.08.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
ZENECA LIMITED 15 Stanhope Gate London W1Y 6LN United Kingdom	GB	GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
SYNGENTA LIMITED Fernhurst Haselmere Surrey GU27 3JE United Kingdom	GB	GB
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:
This is only a change of name and address, no transfer of patent or other rights has occureed. The agent's address has also been changed accordingly.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
R. Chrem
Telephone No.: (41-22) 338.83.38

Copy for the Elected Office (EO/US)

PCT/GB99/02652

PATENT COOPERATION TREATY

09/762769
5660

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

HUSKISSON, Frank, Mackie
Syngenta Limited
Intellectual Property Dept.
Jealott's Hill Research Station
P.O. Box 3538
Bracknell, Berkshire RG42 6YA
ROYAUME-UNI

Date of mailing (day/month/year)
26 July 2001 (26.07.01)

Applicant's or agent's file reference
PPD 50360/WO

IMPORTANT NOTIFICATION

International application No.
PCT/GB99/02652

International filing date (day/month/year)
12 August 1999 (12.08.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
SYNGENTA LIMITED
Fernhurst
Haselmere
Surrey GU27 3JE
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
SYNGENTA LIMITED
Fernhurst
Haslemere
Surrey GU27 3JE
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Dominique DELMAS

Telephone No.: (41-22) 338.83.38

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/31, 15/82, A01H 5/00 | A1

(11) International Publication Number: **WO 00/09696**

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/GB99/02652

(22) International Filing Date: 12 August 1999 (12.08.99)

(30) Priority Data:
9817707.4 13 August 1998 (13.08.98) GB

(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(71) Applicant *(for US only)*: STEWART, Lesley (Personal representative of STEWART, Gordon, Sidney, Anderson, Bernie) [/GB]; 14 James Avenue, Loughborough LE11 5QL (GB).

(72) Inventor: STEWART, Gordon, Sidney, Anderson, Bernie (deceased) (deceased).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FRAY, Rupert, George [GB/GB]; University of Nottingham, School of Biological Sciences, Division of Plant Science, Sutton Bonington Campus, Loughborough LE12 5RD (GB). THROUP, John, Peter [GB/US]; SmithKline Beecham, Pharmaceuticals R & D, UP1345 South Collegeville Road, P.O. Box 5089, Collegeville, PA 19426 (US). WALLACE, Andrew, David [GB/GB]; Unilever Research, Colworth Laboratory, Colworth House, Shambrook, Bedfordshire MK44 1LQ (GB). GRIERSON, Donald [GB/GB]; University of Nottingham, School of Biological Sciences, Division of Plant Science, Sutton Bonington Campus, Loughborough LE12 5RD (GB).

(74) Agents: HUSKISSON, Frank, Mackie et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

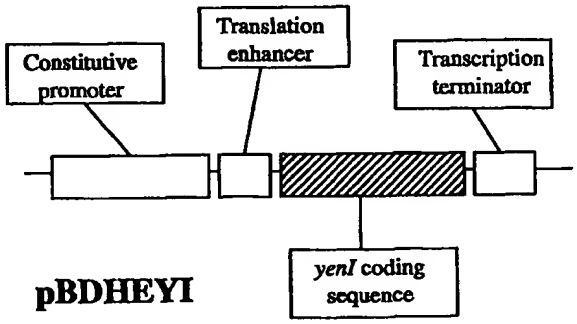
(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: EXPRESSION OF BACTERIAL SIGNAL MOLECULES IN PLANTS

(57) Abstract

The ability of a plant to defend against attack by bacteria, and any virus borne by the bacteria, is enhanced by transforming the plant genome with a gene of bacterial origin which enables the plant to produce a bacterial pheromone, N–acyl–L–homoserine lactone. Such plants also secrete the lactone into the soil enhancing the protective effect of antifungal rhozobacteria.



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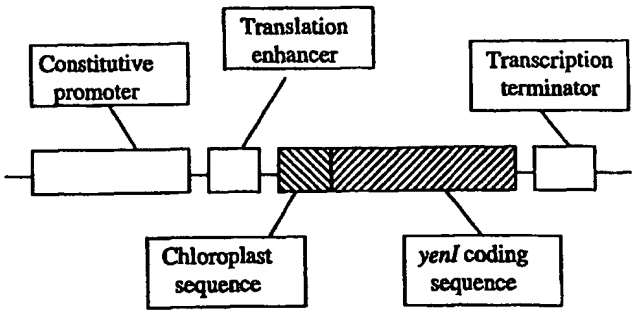

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AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
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CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
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EXPRESSION OF BACTERIAL SIGNAL MOLECULES IN PLANTS

This invention relates to the expression of bacterial signal molecules in plants which allows, for example, modulation of the interaction between plants and infecting or symbiotic bacteria.

The ability of bacteria to respond to environmental cues such as nutrient availability, temperature or pH is critical to microbe success. It is apparent that individual bacteria can also sense the density and state of the local bacterial population of which they are members. This sensing ability, referred to as "quorum sensing", allows a bacterial community to synchronise growth and development and, when the minimum population or "quorum" has been achieved, to initiate a concerted population response. Quorum sensing is thus an example of multicellular behaviour in prokaryotes and regulates diverse physiological processes including bioluminescence, swarming, antibiotic biosynthesis, plasmid conjugal transfer and the production of virulence determinants in pathogens.

The signalling pheromones upon which quorum sensing is based have been identified as *N*-acyl-L-homoserine lactones (reviewed by Swift *et.al.* "Quorum sensing: a population-density component in the determination of bacterial phenotype", *Trends in Biochemical Science,* **21**, 214-219 (1996). *N*-acyl-L-homoserine lactones molecules comprise a homoserine lactone moiety (derived from amino acid metabolism, possibly via S-adenosyl methionine) linked to an acyl sidechain (probably derived from fatty acid synthesis). A number of *N*-acyl-L-homoserine lactones with different acyl side chains have been identified in different bacterial systems where they elicit a wide range of quorum-dependent responses such as swarming, pathogenicity, conjugation or production of colour, light or antibiotics.

Several bacterial species produce the same *N*-acyl-L-homoserine lactone, although in some of the species it may regulate a different biological process. For example, the *luxI* gene product of *Photobacterium fischeri* synthesises *N*-(3-oxohexanoyl)-L-homoserine lactone which regulates bioluminescence in a cell density-dependent manner, whilst the *carI* gene product of *Erwinia carotovora* also produces *N*-(3-oxohexanoyl)-L-homoserine lactone which in this bacterium is responsible for the induction of secreted plant cell wall degrading exoenzymes and of the antibiotic carbapenem. The *cviI* gene of *Chromobacterium violaceum* encodes the enzyme for synthesis of *N*-hexanoyl-L-homoserine lactone which is

structurally very similar to the oxohexanoyl analogue and which induces production of the purple pigment violacein. Inactivation of *luxI, carI* or *cviI* results in loss of the density dependent bioluminescence, virulence or violacein production respectively. The relevant operons can, however, be induced by the addition of an exogenous supply of the *N*-acyl-L-homoserine lactone to the mutant bacteria.

CarI mutants of *Erwinia carotovora* appear to be completely avirulent when tested on tobacco. They can neither macerate plant tissue nor multiply *in planta* because they lack pectin lyase, pectate lyase, polygalacturonase, cellulase and protease. It is pertinent to ask how the expression of these exoenzymes only at high cell density in the wild-type cells may contribute to the success of *Erwinia* as a plant pathogen. It has been suggested that under aerobic conditions, a successful *E.carotovora* infection requires a relatively high inoculum (10^6 - 10^7 c.f.u.) and the progression of the disease is then a competition between bacterial multiplication and development of plant resistance. Thus, the production of macerating enzymes at low cell densities would not give rise to a successful infection, but would result in the induction of the local and systemic plant defence response, which in turn would hamper subsequent infections. Such resistance to *E.carotovora* infection is seen when the plant defence response is artificially induced by the application of salicylic acid.

While not wishing to be bound by any theory as to the manner in which the invention proposed herein operates, the following explanation of the naturally occurring phenomenon of quorum sensing is offered. Using *Photobacterium fischeri* as a convenient example, the expression of two regulatory genes, *luxI* and *luxR*, is necessary for the expression of the genes necessary for bioluminescence. Expression of *luxI* leads to production of the pheromone *N*-(3-hydroxyl)hexanoyl-L-homoserine lactone, the mechanisms by which the lactone is synthesised being largely irrelevant to this discussion. A complex of the pheromone with the protein produced by the *luxR* gene gives a phenotypic response, in the case of *P.fischeri,* bioluminescence. At low population density of bacteria, *luxI* and *luxR* are transcribed at low level and there is insufficient accumulation of the pheromone (*N*-acyl-L-homoserine lactone) to elicit *luxI*-dependent transcription of the operon responsible for visible bioluminescence. It has been suggested that in the absence of sufficient pheromone, and/or a chaperonin known as GroESL, *luxR* is unstable and sensitive to degradation. As the population grows, however, the concentration of the pheromone increases gradually. At a critical level of the pheromone, which represents a critical population density, a complex between *luxR* and the pheromone is thought to bind to a palindromic sequence within the *luxI*

operator thereby activating increased transcription of the operon necessary for increased production of the pheromone and for bioluminescence.

The present invention seeks to provide a method and means of manipulating plant/microbe interactions.

According to the present invention there is provided a method of protecting a plant against bacterial infection and/or virus infection transmitted by bacteria, comprising introducing into the genome of the plant by transformation the ability to synthesise a *N*-acyl-L-homoserine lactone.

Further according to the invention there is provided a method of protecting a plant against bacterial infection and/or virus infection transmitted by bacteria, comprising introducing into the genome of the plant by transformation the ability to synthesise an analogue of *N*-acyl-L-homoserine lactone.

The invention also provides a method of enhancing interaction between an antifungal rhizobacterium and a plant comprising introducing into the genome of the plant by transformation the ability to synthesise the *N*-acyl-L-homoserine lactone naturally produced by the rhizobacterium.

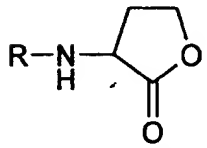
The invention also provides a recombinant plant genome containing a gene construct for *in planta* expression of an *N*-acyl-L-homoserine lactone.

Preferably expression of introduced genes is targeted to plant chloroplasts.

The gene specifying the N-acyl-L-homoserine lactone may be selected from the group consisting of, the *yenI* gene of *Yersinia enterocolitica;* the *cviI* gene of *Chromobacterium violaceum;* the *luxI* gene of *Photobacterium fischeri;* the *carI* gene of *Erwinia carotovora;* the *traI* gene of *Agrobacterium tumefaciens* and the *lasI* and *vsmI* genes of *Pseudomonas aeruginosa.*

Examples of suitable sources of DNAs specifying *N*-acyl-L-homoserine lactones and the acyl group involved are as follows:

Table 1



***N*-acyl-L-homoserine lactone**

Bacterium	Signal Generator	Response Regulator	*N*-acyl-group R
Yersinia enterocolitica	*yenI*	*yenR*	3-oxohexanoyl
Chromobacterium violaceum	*cviI*	*cviR*	3-hexanoyl
Photobacterium fischeri	*luxI*	*luxR*	3-oxohexanoyl
Erwinia carotovora	*carI*	*carR*	3-oxohexanoyl
Agrobacterium tumefaciens	*traI*	*traR*	3-oxo-octanoyl
Pseudomonas aeruginosa	*lasI*	*lasR*	3-oxo-dodecanoyl
Pseudomonas aeruginosa	*vsmI*	*vsmR*	butanoyl

These examples in Table 1 are quoted in Swift *et.al.*, *Trends in Biochemical Science*, **21**, 214-219 (1996).

Table 2 below gives further examples along with references and the appropriate GenBank Accession Numbers.

Table 2

Organism	Signal generator	Response Regulator	Signal Molecule	GenBank Accession number	References
Aeromonas hydrophila	*AhyL*	*AhyR*	unknown	X89469	
Agrobacterium tumefaciens	*TraI*	*TraR*	*N*-(3-oxo)-octanoyl-L-homoserine Lactone (OOHL)	L17024, L22207	Fuqua et.al, 1994; Hwang et.al. 1995
Chromobacterium violaceum	*CviI*	*CviR*	*N*-hexanoyl-L-homoserine lactone (OHL)		Winson,et.al.., (1994)
Enterobacter	*EagI*	unkn wn	*N*-(-3-	x74300	Swift *et al.*, 1993

agglomerans			oxo)hexanoyl-L-homoserine lactone (OHHL)		
Erwinia carotovora subsp carotova	*CarI*	*CarR*	OHHL	U17224, X72891, X74299, X80475	McGowan et.al., 1995
Erwinia stewartii	*EsaI*	*EsaR*	OHHL	L32183, L32184	Beck von Bodman and Farrand, 1995
Escherichia coli	unknown	*SdiA*	unknown	Xo3691	Sitnikov *et al* 1995
Photobacterium fischeri	*LuxI*	*LuxR*	OHHL,OOHL	M19039, M96844, M25752	Meignhen, 1994; Devine *et al*, 1988
Pseudomonas aeruginosa	*LasI*	*LasR*	*N*-(-3-oxo)-dodecanoyl-L-homoserine lactone (OdDHL)	M59425	Winson *et al* 1995;.
	VsmI	*vsmR*	*N*-butanoyl-L-homoserine lactone (BHL), HHL	L08962, U11811, U15644	Winson *et al.*, 1995; Latifi *et al* 1995; Ochsner and Reiser, 1995.
Pseudomonas aureofaciens	*PhzI*	*PhzR*	unknown	L32729, L33724	Wood and Piersen, 1996
Rhizobium leguminosarum	unknown	*RhiR*	*N*(-3-hydroxy)-tetradecanoul-L-homoserine lactone (HtDeHL)	M98835	Fuqua *et al.*, 1994; Gray *et al.*, 1996.
Serratia liquefaciens	*SwrI*	unknown	BHL	U2823	
Aeromonas hydrophila	*ahyI*	*ahyR*	BHL		Swift et al., 1997
Aeromonas salmonicida	*AsaI*	unknown	BHL, *N*-hexanoyl-L-homoserine lactone		Swift *et al.*, 1997
Vibiro	*vanI*	*vanR*	*N*-(3-oxo-		Milton *et al.*,

anguillarum			decan yl)-L-homoserine lactone (ODHL)		1997
Vibrio harveyi	*LuxLM*	*LuxN*	*N*-(3-hydroxy)-butanoyl-L-homoserine lactone (HBHL)	L13940	Meighen, 1994; Bassler *et al.*, 1994.
Yersinia enterocolitica	*YenI*	*YenR*	OHHL,HHL	X76082	Throup *et al.*, 1996.

References: Bassler et.al. *Molecular Microbiology*, **12**, 403-412 (1994)
Beck et.al. *J.Bacteriol.*, 177, 5000-5008 (1995)
Devine et.al. *Biochemistry*, **27**,837-842 (1988)
Fuqua et.al. *J.Bacteriol.* **176**, 269-275 (1994)
Gray et.al. *J.Bacteriol.* **178**, 372-376 (1996)
Hwang et.al. *J.Biotech.* **177**, 449-458 (1995)
Latifi et.al. *Molecular Microbiology*, **17**, 333-343(1995)
McGowan et.al.*Microbiology*, **141**, 541-550 (1995)
Meignhen *Ann. Rev. Genet.* **28**,117-139(1994)
Milton et.al. *J.Bacteriol.* **179**, 3004-3012 (1994)
Ochsner and Reiser *Proc.Natl.Acad.Sci.USA*, **92**, 6424-6428 (1995)
Sitnikov et.al. *Molecular Microbiology*, **17**, 801-812 (1995
Swift et.al. *Molecular Microbiology*, **10**, 511-520 (1993)
Swift et.al. *J.Bacteriol.* **179**, 5271-5281 (1997)
Throup et.al. *Molecular Microbiology*, **17**, 345-356 (1996)
Winson et.al. *Proc.Natl.Acad.Sci USA*, **92**, 9427-9431 (1995)
Wood and Piersen, *Gene* 168, **49**-53 (1996)

Our invention is founded on our reasoning that if the inoculating bacteria were to encounter levels of *N*-acyl-L-homoserine lactone that gave a false indication of the local population size, the course of the ensuing infection would be drastically reduced.

A second aspect of the invention concerns engineering the plant to take advantage of the potential protective effect of antifungal rhizobacteria. There exist in the rhizosphere certain bacteria which are capable of attacking potential pathogenic fungal microorganisms which are also present in the soil, perhaps the best known of which are certain strains of *Pseudomonas fluorescens* and *P. aureofaciens*. But the population of such antifungal bacterial strains in the soil will normally be low and their antifungal activity dependent on the quorum sensing phenomenon to be activated. By imparting to the plant the ability to produce the activator molecule, the *N*-acyl-L-homoserine lactone, appropriate to the antifungal bacteria the antifungal activity may be initiated at low colony size providing earlier than normal protection of the plant against the pathogenic fungi. The rhizosphere-

expressed genes of the *rhiABC* operon of the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*, for example, are regulated by an AHL with a C14 side chain containing hydroxylated carbon in the 3 position and a single carbon-carbon double bond.

Transgenic plants producing an AHL signal molecule enhance the establishment of an antifungal environment on the rhizosphere. This phenomenon would also enable the use of disarmed bacterial strains to be used as crop protection biocontrol agents in conjunction with the AHL-producing transgenic plants.

The invention will now be described in the following Examples

The ability of AHLs to induce changes in neighbouring bacteria was tested in four ways;

(1) the ability of the AHLs to diffuse out of intact leaves was demonstrated by placing intact transgenic leaves on agar and subsequently removing it before overlaying with *C.violaceum* CV026 (see Example 4 below) and the outline of the whole leaf could be seen showing that the AHL diffused out of the leaf surface and not just the cut stem;

(2) being interested in whether the AHLs were only produced in the chloroplasts or whether they could be found in other tissues such as roots, the ability of the AHLs to diffuse from the roots was demonstrated in that AHLs in the vicinity of the roots were able to induce bioluminescence in a recombinant *E,coli* strain carrying an AHL-inducible operon: this showed that the root plastids are competent to suynthesise the AHLs are, alternatively, that the AHLs synthesised in green tissue can be transported to the roots but in either case the roots were clearly capable of signalling to nearby bacteria.

(3) AHL-producing plant tissue is capable of restoring G.graminis growth-inhibiting activity to the disarmed *P.aureofaciens* 30-84 *phzI-* strain (see Example 9 below)

(4) *Erwinia carotovora carI* (*expI*) mutants, which have greatly reduced virulence in their natural host plants were shown to infect transgenic tobacco plants which are producing AHLs (see Example 10 below).

Figure 1 herewith shows the components of the constructs pBDHEYI and pBDHERBYI described in the Examples.

Example 1

Preparation of pBDHEYI

pBDHEYI was constructed by fusing the alfalfa mosaic virus (AMV) translation enhancer sequence from pBI526 (Datla et.al., *Plant Science* **94**, 139-149 (1993)) to the *yenI*

coding sequence from *Yersinia enterocolitica*. The *yenI* sequence had previously been amplified by PCR to create an *Nco*I site overlapping the translation initiation sequence. This changed the second amino acid from leucine to valine but did not affect the ability of the encoded enzyme to synthesise *N*-acyl-L-homoserine lactones in a bacterial assay. The AMV/*yenI* fusion was cloned on a *Bgl*II/*Bam*HI fragment into the *Bam*HI of pDH51 (Pietrzak *et.al.*, *Nucl. Acids Res.* **14**, 5857-5868(1986)) to give pDHEYI. An *Eco*RI fragment of pDHEYI was cloned into the *Eco*RI site of pBIN19 (Bevan, *Nucl. Acids Res.* **12**, 8711-8721 (1984)) to give pBDHEYI.

Example 2

Preparation of pBDHERBYI

pBDHERBYI was constructed by fusing the petunia SSU611 ribulose bisphosphate carboxylase small subunit (*rbcS*) chloroplast targeting sequence (Dean et.al. *Mol, Gen. Genet.*, 206, 465-474 (1987)) to the AMV translation enhancer sequence of pBI526. An *Nco*I site was engineered to overlap the initiating ATG codon of *rbcS*. An *Sph*I site was engineered to overlap the initiating ATG codon of *yenI* and the *yenI* coding sequence cloned into the *Sph*I site of the SSU611 fragment. This site spans the cleavage site of the encoded chloroplast transit peptide. The AMV/*rbcS*/*yenI* fusion was cloned on a *Bgl*II/*Bam*HI fragment into the *Bam*HI site of pDH51 to give pDHERBYI. An *Eco*RI fragment from pDHERBYI was cloned into the *Eco*RI site of pBIN19 to give pBDHERBYI.

The rationale for producing pBDHERBYI and believing that it would be active in chloroplasts was as follows: in *E.coli* homoserine lactone is not produced by mutants of the threonine biosynthetic pathway that are blocked prior to homoserine synthesis but is produced by those mutants when supplied with an exogenous source of homoserine. However, *TraI*, the *N*-acyl-L-homoserine lactone biosynthetic enzyme in *Agrobacterium tumefaciens*, has been found to utilise *S*-adenosylmethionine and not homoserine as a substrate *in vitro*. There is also evidence for the acyl moiety being derived from fatty acid biosynthetic intermediates. In plants the enzymes of the threonine biosynthetic pathway are located in the chloroplast and this organelle is also active in fatty acid metabolism. Therefore the chloroplasts may be expected to contain the necessary precursors for *N*-acyl-L-homoserine lactone synthesis by *yenI* and more closely approximate to the environment in which *yenI* is normally active than would be the cytoplasm.

Example 3

Generation of Transgenic Plants

Construct pBDHEYI.for Example 1 and pBDHERBYI from Example 2 were transferred to the *Agrobacterium tumefaciens* strain LBA 4404 and used to transform tobacco leaf discs according to standard protocol (Draper et.al., pages 69-160, In Plant Genetic Transformation and gene expression: a laboratory manual; Draper et.al. (Eds) Blackwell Scientific Publications, London (1988)).

The transgenic status of the resulting kanamycin positive explants was confirmed by Southern analysis (data not given)

Example 4

Complementation of Violacein Production

Leaf segments of the transgenic plants produced in Example 3 were tested for their ability to synthesise *N*(3-oxohexanoyl)-L-homoserine lactone or a related analogue.

A transgenic tobacco leaf was placed in an agar plate overnight. The leaf was then removed and the *cviI* mutant of *Chromobacterium violaceum* spread over the plate. Violacein production by the bacteria could be seen where the *N*-(3-oxohexanoyl)-L-homoserine lactone had diffused out of the leaf and into the agar.

Two leaf segments tested positive as indicated by the ability of a diffusible product to complement *C.violaceum*, inducing the production of the purple pigment violacein by the bacteria.

Example 5

Complementation of *carI*

Construct pBDHERBYI (Example 2) was transferred to the *Agrobacterium tumefaciens* strain LBA 4404 and transformed into tobacco. Leaf segments were tested for their ability to synthesise *N*(3-oxohexanoyl)-L-homoserine lactone or a related analogue.

An untransformed control and a transgenic BDHERBYI tobacco leaf were inoculated with *Erwinia carotovora* mutant for *carI*. The bacteria were applied at a high culture density (OD600 of 2.5) in a volume of 10 µl to a small wound site made with a hypodermic needle. A second BDHERBYI leaf was mock inoculated with bacterial culture medium alone.

The leaves were inspected after four days. The untransformed control and the mock inoculated leaf remained substantially unchanged. The sample inoculated with *E.carotovora* displayed advanced disease symptoms demonstrating that the pathogen can perceive and respond to the *N*-acyl-L-homoserin lactone being made by the transgenic plant.

Example 6

Complementati n f *luxI*

Following a similar protocol as described above, the *luxR* *N*-acyl-L-homoserine lactone response regulator and the *lux* operon (minus *luxI)* of *Pseudomonas fischeri* was inserted into *E.coli*. When transgenic tobacco carrying the BDHERBYI construct was challenged with the *E.coli*, bioluminescence was induced in the bacteria demonstrating that the *luxR* gene was able to respond to the *N*-acyl-L-homoserine lactone produced by the plant.

Twenty-nine tobacco plants that were independently transformed with either BDHERBYI or BDHEYI were challenged with *C.violaceum* mutant for *cviI* (Example 3) and *E.coli* carrying an *N*-acyl-L-homoserine lactone-inducible *lux* operon. Table I summarises the results.

TABLE 1

	Number of plants				
	Positive reaction		**Negative reaction**		
Construct	***cviI***	***luxI***	***cviI***	***luxI***	**Total**
BDHERBYI	8	8	5	5	13
BDHEYI	0	0	16	16	16

Example 7

Extraction and TLC analysis of AHLs

For thin-layer chromatographic analysis, transgenic plant extracts were made by grinding two grams of plant tissue to a fine powder in liquid nitrogen and mixing the frozen powder with 200ml of warn distilled water. After five minutes, solid matter was filtered off and the filtrate extracted with an equal volume of ethyl acetate. The ethyl acetate layer was then dried over anhydrous magnesium sulphate, filtered and evaporated to dryness. The residue was taken up in 500 µl of acetonitrile and 20µl of this was applied to a C18 reverse phase TLC plate (Merck). A similar extract from an untranformed control plant was also spotted on to the plate. *N*-hexanoyl-L-homoserine lactone (HHL) ($1x10^{-8}g$) and *N*-(3-oxohexanoyl) -L homoserine lactone (OHHL) ($5x10^{-7}g$) were applied as standards and the chromatogram developed with methanol/water (60:40 vol/vol) as running solvent (Shaw P.D. *et.al.* Proc.Natl.Acad,Sci. USA 94: 6036-6041 (1997)). After drying, AHLs were located on the TLC plate by overlaying *C.violaceum* strain CV026 in top agar as described by McClean *et.al.* (Microbiology-UK, 143: 3703-3711 (1997). After 16 hours growth at 28°C the presence of AHLs was indicated by localised violacein production.

Two different molecules with R_f values identical to the synthetic HHL and OHHL standards were observed.

Example 8

HPLC and LC-MS Analyses

For HPLC and LC-MS analyses, transgenic plant extracts were made by grinding the issue in ethyl acetate. The supernatant was taken and the plant residue re-extracted with ethyl acetate, the supernatants pooled and the process repeated until the plant residue was white/brown n colour and free of chlorophyll. The ethyl acetate layer was separated from a small plant-derived aqueous layer and dried over anhydrous magnesium sulphate, filtered, and evaporated to dryness. The residue was resuspended in 500 µl of methanol, this was brought to 60% methanol with sterile distilled water and placed at -20°C overnight to precipitate out the majority of the chlorophyll. After pelleting any solid matter by centrifugation in a bench-top microfuge, the AHL-containing supernatant was partitioned against 10 volumes of ethyla acetate and the organic phase evaporated to dryness. The residue was taken up into 500µl of acetonitrile. For both LC-MS and HPLC analyses linear gradients of acetonitrile in water were run (20-100% over 32 minutes) as described by Camara *et.al.* In Methods in Microbiology: Bacterial Pathogenesis Vol. 27: 319-330, Williams *et.al.* (Eds) (1998). OHHL and HHL eluted at 9 minutes and 13.5 minutes respectively.

The presence of HHL and OHHL, detected in TLC analysis, were confirmed.

Example 9

Assay for restoration of activity to *P.aureofaciens* mutant

Leaf material from transgenic and non-transformed control plants were placed in wells cut in a potato dextrose agar plate (Oxoid). *P.aureofaciens* strain 80-84I (*phzI*$^-$) was inoculated adjacent to the wells and the plates incubated for 24 hours at 22°C. The *G.graminis* var. tritici was then introduced on the opposite side of the plate and the whole incubated for a further four days.

The antifungal activity of the *P.aureofaciens phzI*$^-$ strain against the *G.graminis* was found to have been restored.

Example 10

Assay for restoration of virulence to *Erwinia carotovora* avirulent mutant

Untransformed and control BDHERBYI tobacco leaves were inoculated with the avirulent E.carotovora mutant PNP22 (Bainton *et.al.*, Biochem.Journal, **288**: 997-1004

(1992) and also Jones *et.al.*, The EMBO Journal, 12: 2477-2482 (1993)) The bacteria were applied at high culture density (OD600=2.5) in a volume of 10µl to a small wound site made with a hypodermic needle.

Normally these *Erwinia* mutants are avirulent in the tobacco system, in which they can neither macerate plant tissue nor multiply *in planta* because they are defective in the production of plant cell-wall-degrading enzymes pectin lyase, pectate lyase, polygalacturonase, cellulase and protease. The regulated expression of plant cell wall-degrading enzymes only at high density in wild-type bacteria may contribute to the success of *Erwinia* as a plant pathogen. Under aerobic conditions, *E.carotovora* infection only occurs when the bacteria has reached sufficiently high population densitiy such that disease progression depends on competition between bacterial multiplication and the plant host defences. Thus the production of macerating enzymes at low cell densities would not give rise to a successful infection, but would result in the premature induction of the local and systemic plant defence response, which in turn would hamper subsequent infection. Thus, if the infecting pathogen were to encounter AHL levels that gave a false indication of the local bacterial population size the course of the ensuing infection will be substantially reduced as the plant is able to mount a successful defence to a weak attack.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

CLAIMS

1. A method of protecting a plant against bacterial infection and/or virus infection transmitted by bacteria, comprising introducing into the genome of the plant by transformation the ability to synthesise a *N*-acyl-L-homoserine lactone.

2. A method of protecting a plant against bacterial infection and/or virus infection transmitted by bacteria, comprising introducing into the genome of the plant by transformation the ability to synthesise an analogue of *N*-acyl-L-homoserine lactone capable of competing with the *N*-acyl-L-homoserine lactone secreted by infecting bacteria for *N*-acyl-L-homoserine lactone receptor sites therein.

3. A method of enhancing interaction between a rhizobacterium and a plant comprising introducing into the genome of the plant by transformation the ability to synthesise the *N*-acyl-L-homoserine lactone naturally produced by the rhizobacterium.

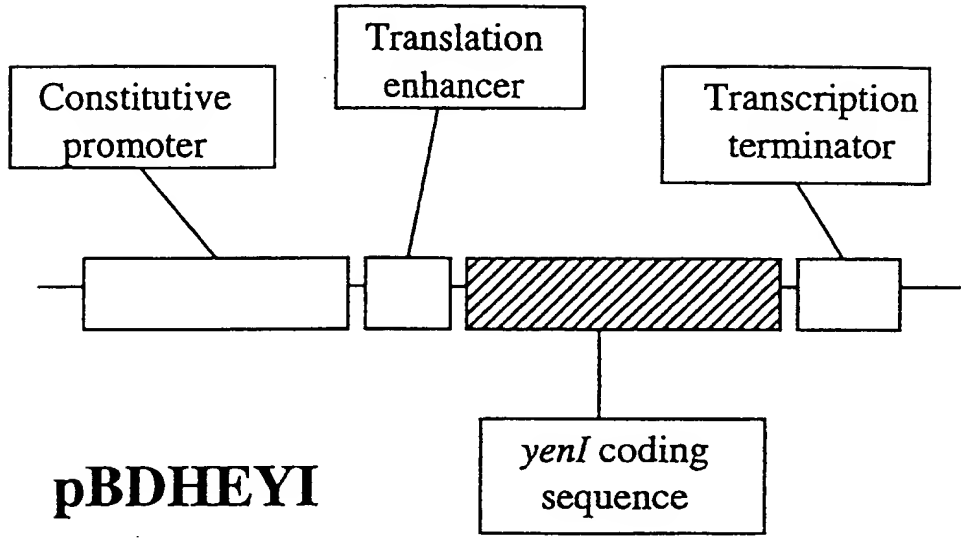
4. A method as claimed in any of claims 1 to 3 in which the gene expressing the N-acyl-L-homoserine lactone is selected from the group consisting of, the *yenI* gene of *Yersinia enterocolitica;* the *cviI* gene of *Chromobacterium violaceum;* the *luxI* gene of *Photobacterium fischeri;* the *carI* gene of *Erwinia carotovora;* the *traI* gene of *Agrobacterium tumefaciens* and the *lasI* and *vsmI* genes of *Pseudomonas aeruginosa.*

5. A recombinant plant genome containing a gene construct for *in planta* expression of an *N*-acyl-L-homoserine lactone and/or the response regulator thereof.

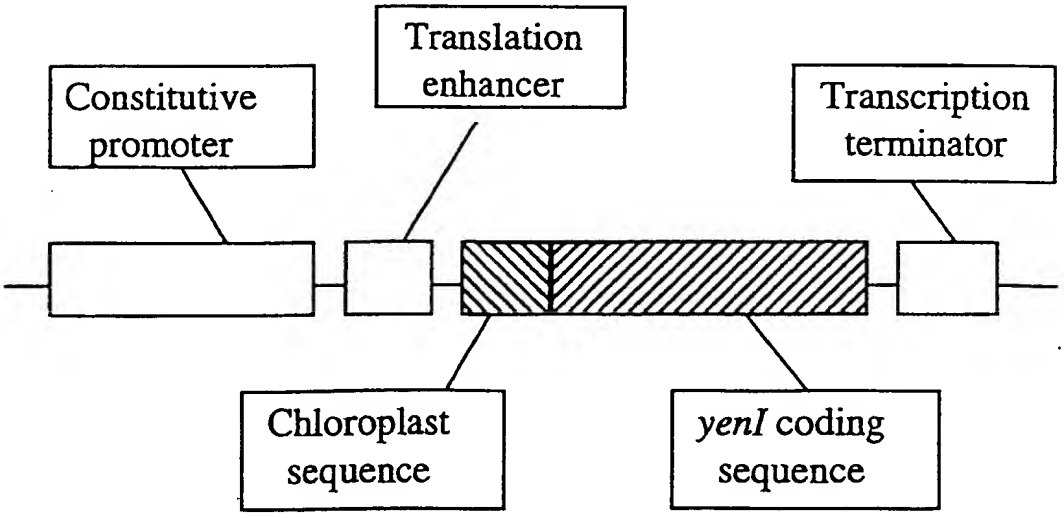
6. A genome as claimed in claim 5 in which expression of the said *N*-acyl-L-homoserine lactone is targeted to plant chloroplasts.

1/1

Figure 1



pBDHEYI



pBDHERBYI

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/02652

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 195 48 301 C (DUERING KLAUS DR) 27 February 1997 (1997-02-27) the whole document ---	1-6
A	WO 97 48719 A (BECKERMAN JANNA L ;TEXAS A & M UNIVERSITY SYST (US); ZHANG LEI (US) 24 December 1997 (1997-12-24) the whole document ---	1-6
A	WO 96 29392 A (UNISEARCH LTD ;KJELLEBERG STAFFAN (AU); STEINBERG PETER (AU); NYS) 26 September 1996 (1996-09-26) the whole document ---	2

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
2 November 1999	17/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Kania, T

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